# REAL TIME BIODETECTION OF INDIVIDUAL PATHOGENIC MICROORGANISMS WITH A RUGGED, SUB-BRIEFCASE-SIZED SYSTEM

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## ABSTRACT

The objective of this research is to test the feasibility of an innovative approach to rare cell detection: using a CCD to image an entrained flow through a rectangular glass tube. This approach is especially useful for the detection of rare cells where a high volumetric flow rate is desired. We present the results of our work with Nile red labeled 1-micron polystyrene beads as labeled cell simulants. This technique has key advantages over current alternatives, including: (1) high volumetric flow rate, 2) capability of detecting single microorganisms (3) automatic operation, and (4) easy implementation in a rugged, portable system.

## INTRODUCTION

Rapid biodetection of specific pathogenic microorganisms at very low concentrations usually requires immunofluorescence detection with an epifluorescence microscope or conventional flow cytometer. We present an immunofluorescence detection approach that lends itself to low-cost detection in a rugged, miniature package. While conventional flow cytometers are essential for making accurate photometric measurements of light scattering from cells, simpler means may be used where only cell identification is necessary. This research was based on use of a two-dimensional or panoramic CCD (charge-coupled device) detector, which allows for a much greater signal-to-noise ratio (S/N) than conventional photomultiplier or photodiode systems.

A second important achievement of this research was successful demonstration of a “sheathless” (or core-flow-only) flow cell, providing for simpler construction and operation of the flow cytometer and

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potentially far greater volumetric flow rate than the conventional cylindrical core/sheath flow device. This was accomplished by using a "*ribbon flow*": flowing the sample through a rectangular, transparent tube, large enough to inhibit clogging, yet small enough to enable imaging all of the target particles flowing through it.

## APPROACH

The SoftRay approach to biodetection is an unconventional form of flow cytometry (patents pending) based on:

- a sample flow entrained by a rectangular glass tube to deliver the total volume of solution being sampled into the laser beam for detection,
- fluorescent labels to provide high selectivity, and
- use of a CCD with time delayed integration (TDI)[1] to provide the highest signal-to-noise (S/N) and the possibility of spectral discrimination for simultaneous identification of more than one species. This technique is called time delayed integration because the CCD is read out continuously, one line at a time, in sync with a moving image, as opposed to reading the entire image at once. TDI allows for detection in real time, with a reduction in background noise, as explained later.

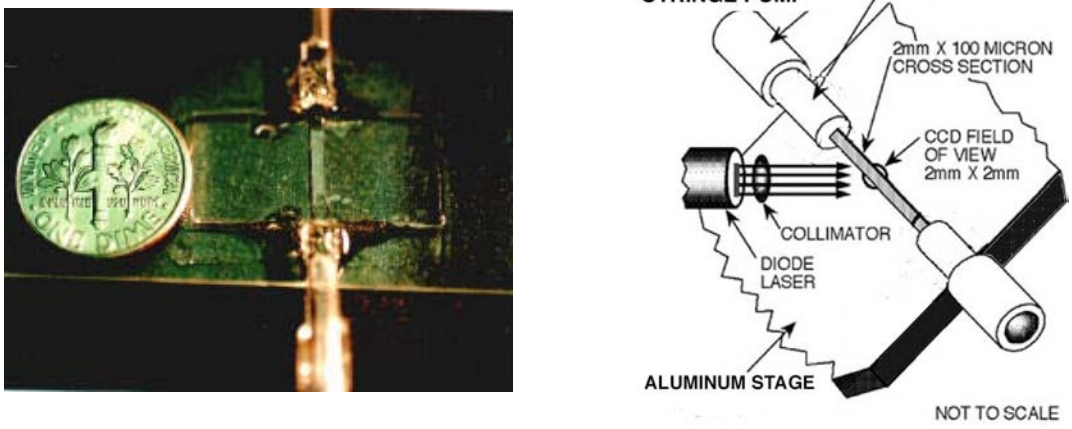


Figure 1. A schematic of the SoftRay flow cytometer used to test the concepts of TDI with a ribbon-flow geometry. On the left is an early version of our miniature flow cell, consisting of a 1 mm x 50 μm flow channel mounted on a microscope slide and connected to Teflon tubing through two short 3 mm glass tubes. A drawing of our current design is shown on the right. This version of our instrument employs a 4.0 mW Nd:YAG laser, a 2.0 mm x 100 μm rectangular flow tube, a 4x-microscope objective, an inexpensive, uncooled CCD camera, and an IBM-compatible PC for data collection. An image made from a similar flow cell is shown in Fig. 4.

In this research, a fully operational TDI/CCD flow cytometry apparatus was assembled and tested with 1-micon Nile red fluorescent microbeads (Molecular Probes, Eugene, OR). The flow cell and the laser source are shown in Fig. 1. This device enabled the flow cytometry feasibility demonstration and established the suitability of the TDI/CCD flow cytometer to *effectively detect single pathogenic microorganisms*. Emphasis was placed on signal-to-noise enhancement. In this detection system, SoftRay successfully used "sheathless" ribbon flow geometry. Fluid is transported through a transparent rectangular flow tube that is large enough (2mm x 100 μm) to prevent clogging (Fig. 1). Our imaging technique allows measurement of individual cells while they transit this flow cell.

A fundamental difficulty with conventional flow cytometry is embodied in the competing requirements of high flow rate, to provide near-real-time detection of microorganisms, and high sensitivity. High sensitivity is predicated on having a fluorescing microorganism in the detection region long enough to provide for a high S/N ratio, and having an optical design that limits background from scattering and fluorescence from unbound dye, which can dominate the background. The optimal device is a flow cytometer with a small excitation beam, a high flow rate, and detection electronics that allow for collection of enough photons from a microorganism for reliable detection. Time delayed integration with a CCD camera can provide such a system. TDI allows for detection in real time (in seconds) but with a significant reduction in background noise, as explained below and in Fig. 3. The time delayed integration (TDI) technique was first discussed by Barbe[1] and developed by Wright and Mackay for astronomy[3].

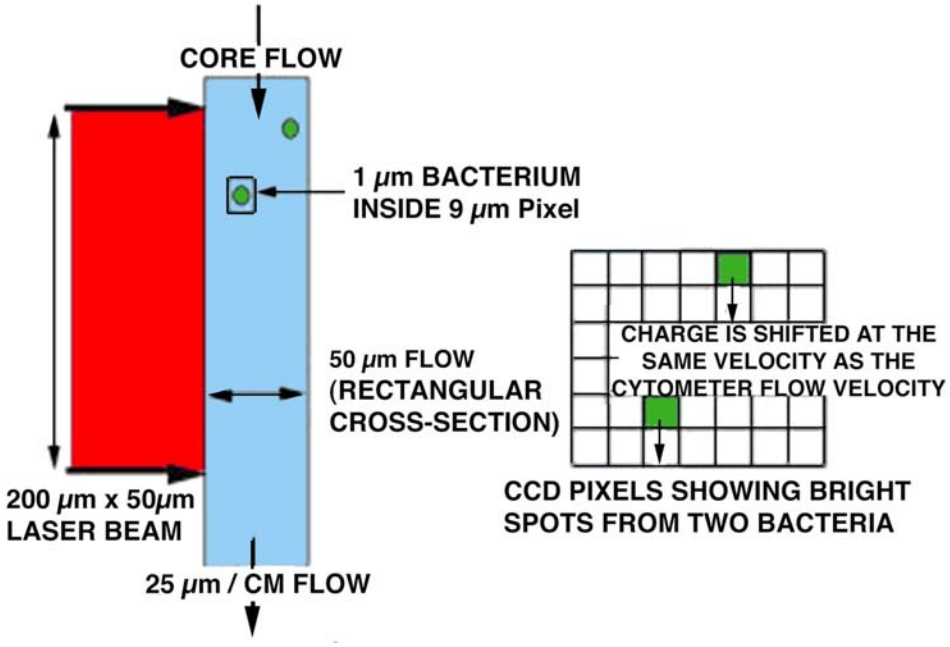


Figure 2. The detection signal-to-noise is improved with imaging TDI. Left panel: two bacteria (circular) are shown in the core flow illuminated by a laser beam from the left. Right panel: the image, at the CCD surface, where pixels illuminated by the two previous bacteria are shaded. The charge packets associated with these illuminated spots are shifted down the CCD at the same rate as the bacteria move in the cytometer flow. The result is that a moving volume 9 μm × 9 μm × 50 μm is imaged onto the CCD and read out as a pixel. Using a single, conventional detector, a volume 200 μm × 50 μm × 50 μm is detected. This would produce the same intensity contribution from the bacterium as would a CCD, but with more than 100 times the background intensity.

Conventionally, a CCD image is made by opening a shutter, exposing the entire CCD array to an image, closing the shutter, and reading the device. After an exposure, a charge distribution exists across the CCD, with each pixel carrying an electronic charge proportional to the light having fallen on that pixel during the exposure. After the exposure, the charge is transferred, row by row, into a serial transport register. After each row transfer, the individual pixels are transferred, one at a time, through an on-chip output amplifier and digitized. The readout and digitization are generally performed as quickly as possible (in milliseconds), under existing noise constraints.

With the TDI technique, each row is shifted more slowly, to synchronize the pixel shift rate with the rate at which the image moves across the CCD. The CCD is not shuttered, but is read out continuously. Image smearing is avoided by moving the image across the CCD at the same rate that the CCD charge is being shifted (Fig. 3). For the TDI flow cytometer described here, the chip will be oriented so that the charge packets are shifted down, synchronously with the passage of bacteria through the detection region.

We assembled a CCD flow cytometer that demonstrated the practical and economic feasibility of our proposed method for real time detection of pathogenic microorganisms using an imaging CCD camera with a novel time delayed integration (TDI) technique (where the CCD imaging is synchronized with the flow cytometer stream velocity) to image and count individual microorganisms.

We initially chose not to test TDI/CCD flow cytometry with a conventional sheath/core flow cytometer. Instead, we based our initial proof-of-concept device on a flow cell consisting of 1-mm × 50 μm rectangular glass tubing. This eventually evolved into an improved, innovative, sheathless flow cytometer, which appears to be well suited to TDI/CCD flow cytometry. Without a sheath flow, the cytometer has been greatly simplified. In addition, with a ribbon flow geometry flow cytometer, the cross-sectional area, and therefore the sampling rate, can be easily increased by a factor of 10-100 over conventional flow cytometry. Side-illumination of the rectangular ribbon flow with a laser diode with a rectangular beam allows one to maximize the energy density of photons within the irradiated region of the flow. The dimensions of the ribbon flow are determined by the width of the CCD field of view and the depth of focus of the optics, which are in turn set to balance S/N and flow throughput.

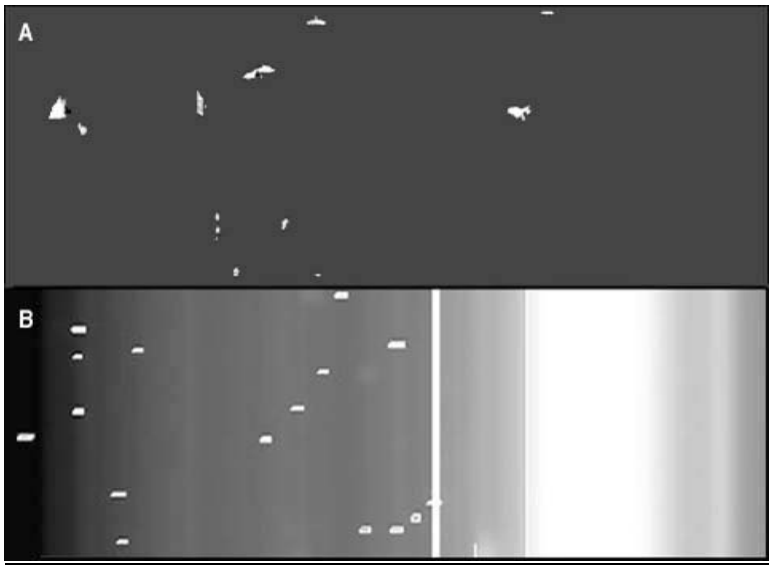


Figure 3. Initial tests with TDI were made with fluorescent 6.5-μm microspheres. These latex spheres were illuminated with a 2-mW HeNe laser and fluoresced at 671 nm. A. An image made through a 670-nm narrowband filter shows only the fluorescing microspheres. B. In unfiltered light, bubbles and other defects in the optical epoxy at the rectangular glass interface appear as streaks in TDI images.

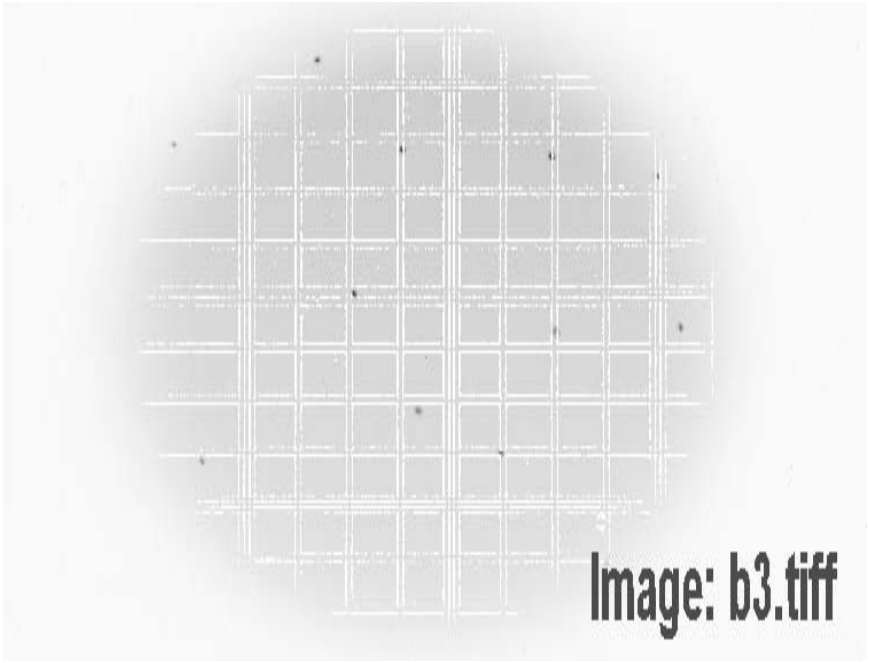


Figure 4. Fluorescing Nile red labeled polystyrene beads are seen under a Petroff-Hausser slide with an Olympus BH-2 epifluorescent microscope with 488 nm illumination. The negative fluorescence image is overlaid with a white light image of the Petroff-Hausser grid to facilitate concentration determination.

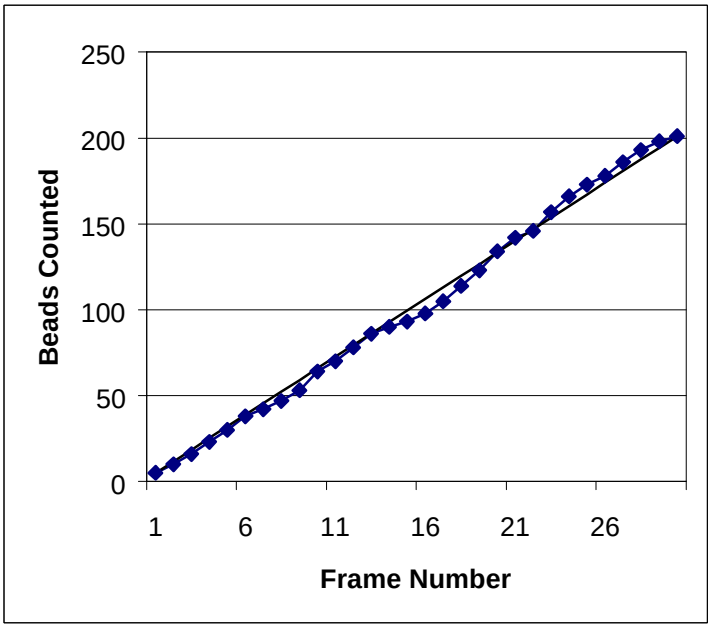


Figure 5. The cumulative number of beads per TDI image frame is shown for a 6.9 microliter sample counted by the TDI/CCD flow cytometer. This figure illustrates the consistency in counts from image to image.

We successfully performed the efficiency tests on our prototype flow cytometer configuration. Performance tests were made with 1-micron polystyrene beads labeled with Nile red dye. Known concentrations of calibration beads were prepared from a stock solution, then diluting this solution at a

number of lower concentrations. These concentrations were measured using a Petroff-Hausser slide with an epifluorescent microscope (Olympus BH-2). Petroff-Hausser measurements were made with an Electrim CCD camera. First a white-light image of the Petroff-Hausser slide was made. Then a fluorescent image was made of the bead dilution, illuminated at 488-nm. The white-light grid was digitally laid over the fluorescent image (Figure 4).

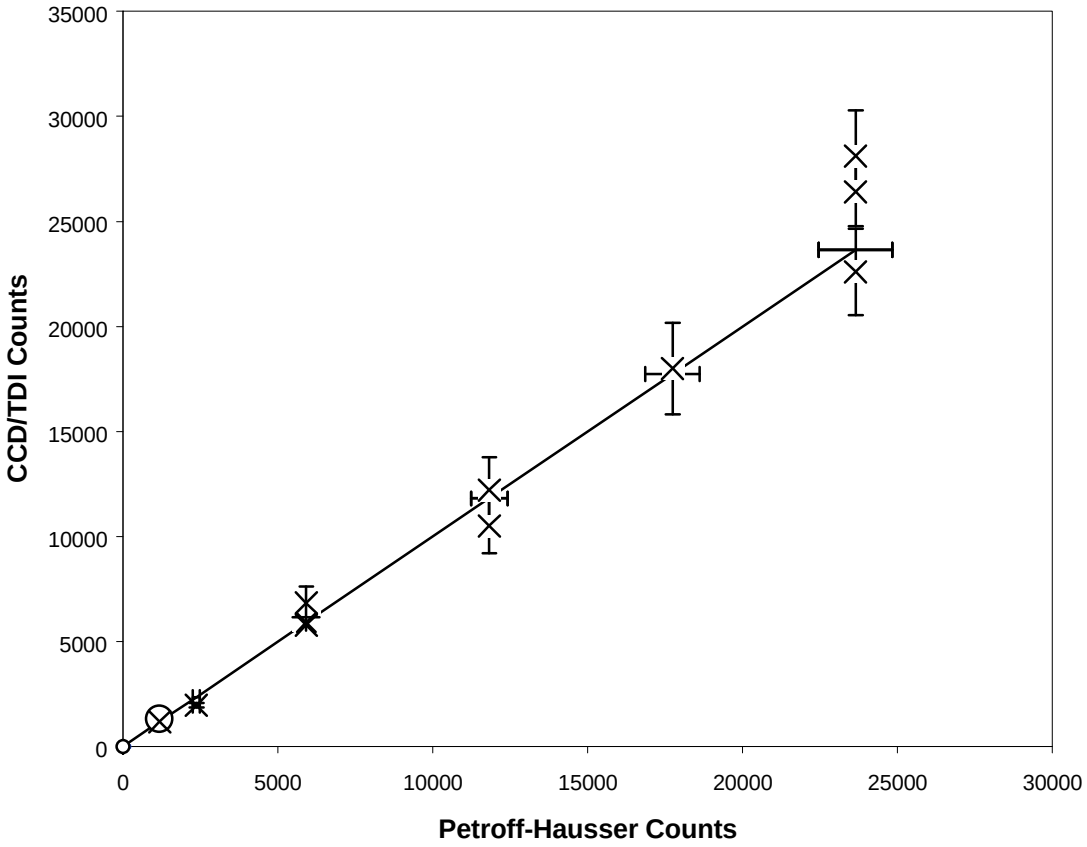


Figure 6. Comparison of CCD/TDI counts of Nile red labeled polystyrene bead dilutions with Petroff-Hausser counts (see Figure 5) on 6.9 microliter samples. Each of the clusters of measurements corresponds to a single dilution. Petroff-Hausser counts fall along the solid line. Horizontal error bars are the 1-$\sigma$ error bars for the Petroff-Hausser counts. "X"s correspond to the CCD/TDI measurements. Vertical error bars represent the 1-$\sigma$ error bars for the CCD/TDI counts. Each CCD/TDI measurement is the result of counting 30 frames, corresponding to 6,9 microliters of fluid. A sample of buffer solution with no beads was run as a null sample and yielded no counts. The result is a point plotted at the origin.

## CONCLUSIONS

We counted the fluorescing beads in a CCD/TDI flow cytometer by TDI imaging the flow continuously as 1 ml of sample traversed the flow cell, then counting fluorescing cells from 30 of the TDI images by computer, corresponding to 6.9 microliters (230 nanoliters/frame). As each bead detection is based on imaging a single bacterium at high (>20) S/N ratio, we have shown that our technique is capable of single cell detection even at low (<10/ml) microbe concentrations.

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